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Separate photosensitizers mediate degradation of the 32-kDa photosystem II reaction center protein in the visible and UV spectral regions

(*Spirodela oligorrhiza*/protein turnover/quinone/chloroplasts)

BRUCE M. GREENBERG*†, VICTOR GABA*, ORA CANAANI‡, SHMUEL MALKIN‡, AUTAR K. MATTOO§, AND MARVIN EDELMAN*

Departments of *Plant Genetics and ‡Biochemistry, The Weizmann Institute of Science, Rehovot 76100, Israel; and §Plant Molecular Biology Laboratory, Beltsville Agricultural Research Center, U.S. Department of Agriculture, Agricultural Research Service, Beltsville, MD 20705

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ABSTRACT **A component of the photosystem II reaction center, the 32-kDa protein, is rapidly turned over in the light. The mechanism of its light-dependent metabolism is largely unknown. We quantified the rate of 32-kDa protein degradation over a broad spectral range (UV, visible, and far red). The quantum yield for degradation was highest in the UV_B (280–320 nm) region. Spectral evidence demonstrates two distinctly different photosensitizers for 32-kDa protein degradation. The data implicate the bulk photosynthetic pigments (primarily chlorophyll) in the visible and far red regions, and plastoquinone (in one or more of its redox states) in the UV region. A significant portion of 32-kDa protein degradation in sunlight is attributed to UV_B irradiance.**

Photosynthetic oxygen evolution is catalyzed by photosystem II (PSII), a complex of several proteins and pigments located in the chloroplast membrane (1, 2). The PSII reaction center contains three proteins—the 32-kDa protein, D_2, and cytochrome b_{559}—as well as bound chlorophylls, pheophytins, quinones, and nonheme iron (3, 4). The 32-kDa protein (also known as D_1 and the Q_B protein) has received considerable attention because it is a major product of the chloroplast protein synthesizing machinery (5, 6), is rapidly turned over as a function of the visible light intensity (7), and is the direct target for PSII herbicides such as atrazine and diuron (8, 9). These herbicides block electron transport through PSII (10) and inhibit 32-kDa protein degradation (7) by displacing a liganded quinone (Q_B) from the protein (11). Indeed, the primary site of 32-kDa protein cleavage (12) maps to a phylogenetically conserved domain (5), which is adjacent to the quinone and PSII herbicide binding pocket (13). The cleavage site is contiguous with an α-helix destabilizing region (12) common to many rapidly degraded proteins (14).

Although considerable structural and functional information has been amassed concerning that region of 32-kDa protein where primary light-dependent cleavage of the protein occurs, little is known of how the cleavage site becomes activated by light. Neither the photosensitizer nor any photoactive intermediate has been identified. We previously concluded that phytochrome is most likely not a photoreceptor for degradation of 32-kDa protein in the visible or far red regions of the spectrum (15). On the other hand, it has been speculated (15, 16) that damage mediated by a semiquinone anion radical [normally formed in the Q_B pocket during photosynthesis (17, 18)] might play a role in promoting degradation of the protein. In this regard, we noted that quinone, semiquinone anion radical, and quinol all have characteristic UV spectra (19) and, furthermore, that UV irradiation inhibits PSII electron flow (20–23). We thus decided to determine whether UV-absorbing factors are involved in 32-kDa protein turnover.

This report demonstrates that 32-kDa protein is rapidly degraded under UV radiation. Several lines of evidence indicate that distinctly different photosensitizers activate the degradation process in various spectral regions. The data implicate bulk photosynthetic pigments in the visible and far red regions and quinones in the UV region.

MATERIALS AND METHODS

Plant Growth. Axenic cultures of *Spirodela oligorrhiza* (Kurtz) Hegelm were phototrophically cultured on half-strength Hutner's medium (24) under 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of photosynthetic photon flux provided by cool-white fluorescent light.

Radiation Sources. Sources are given in the hierarchy: wavelength (half-power band width); source, 254 nm (1 nm, Hg emission line), 300 nm (40 nm), and 350 nm (30 nm); Rayonet photoreactor bulbs, 280 nm (30 nm); 900-W Xe lamp with monochrometer (Schoeffel), 313 nm, 366 nm, and 405 nm (1 nm, Hg emission lines); 250-W Hg lamp fitted with GG19 + UG11 (Schott), UG1 + WG360 (Schott), and GG385 (Schott) + 7–59 (Corning) filters, respectively; 429 nm, 447 nm, 560 nm, 660 nm, 716 nm, and 731 nm (all 10–20 nm); 250-W tungsten-halogen projectors fitted with a heat-absorbing glass plus the appropriate interference filter (Schott, Balzers, or Detric). Visible light was generated with cool-white fluorescent bulbs. Photon fluence rates were measured with a LiCor quantum flux meter (visible and far red) or a potassium ferrioxalate actinometer (UV) (25). For far red light measurements, the LiCor quantum flux meter was calibrated with a LiCor spectroradiometer.

Other Methods. Conditions for *in vivo* radiolabeling, isolation of membrane proteins, their fractionation by SDS/PAGE, quantification by densitometry, and determination of *in vivo* absorptance (1 − transmittance) and photosynthetic activity spectra are described in the figure legends.

RESULTS AND DISCUSSION

Turnover of 32-kDa Protein in UV Light. The 32-kDa protein is rapidly turned over in visible light (400–700 nm), where both PSII and photosystem I (PSI) are functional (7), and in far red light (>700 nm), where PSI is predominantly active (15). The results presented in Fig. 1*A* show that

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Abbreviation: PSII, photosystem II.

†To whom reprint requests should be sent at present address: Department of Biology, University of Waterloo, Waterloo, ON N2L 3G1, Canada

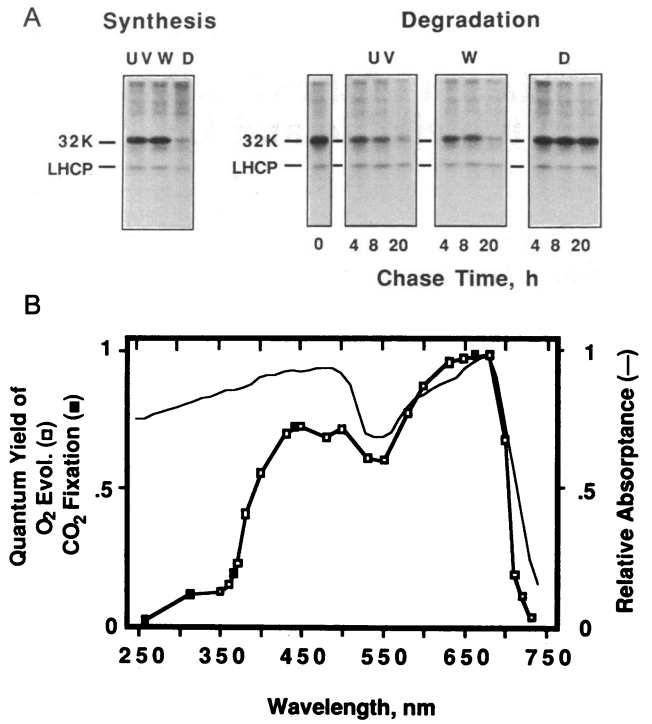

FIG. 1. (*A*) *In vivo* turnover of 32-kDa protein in UV-irradiated plants. To observe synthesis of 32-kDa protein, *Spirodela* plants were radiolabeled with [^{35}S]methionine for 1 hr (15). Radiation conditions were as follows: visible light (lane W), 6 μmol·m^{-2}·s^{-1}; 300-nm UV radiation (lane UV), 6 μmol·m^{-2}·s^{-1}; darkness (lane D). To observe degradation of 32-kDa protein, *Spirodela* plants were radiolabeled with [^{35}S]methionine for 3 hr under 25 μmol·m^{-2}·s^{-1} visible light and chased in 1 mM methionine for the times indicated (15). Radiation conditions for the chase experiments were as follows: visible light (lanes W), 6 μmol·m^{-2}·s^{-1}; 300-nm UV radiation (lanes UV), 6 μmol·m^{-2}·s^{-1}; darkness (lanes D). For both synthesis and degradation experiments, plants were removed at the times indicated and their membrane proteins were fractionated by SDS/PAGE (26). Gels were loaded on an equal protein basis and the separated proteins were visualized by fluorography. Positions of 32-kDa protein and the light-harvesting chlorophyll *a*/*b* binding protein (LHCP) are indicated. (*B*) *In vivo* absorptance and photosynthetic activity spectra for *Spirodela*. The absorptance spectum (—) of intact plants in water was determined from 250 nm to 740 nm using a Cary 15 spectrophotometer equipped with an integrating sphere. Absorptance (1 − transmittance) is the fraction of the incident beam absorbed by the plant. The data were normalized to a peak value of 1.0 at 675 nm. The photosynthetic activity spectrum was determined at several wavelengths from 254 nm to 740 nm. The quantum yield of oxygen evolution (■) was measured by photoacoustic spectroscopy (27) and was normalized to a peak value of 1.0 at 670 nm. The quantum yield of CO_2 fixation (□) was measured after incubation of plants for 1 hr in growth medium containing [^{14}C]bicarbonate (4 μCi/ml; 1 Ci = 37 GBq) at a photon flux of 6 μmol·m^{-2}·s^{-1} at the wavelengths indicated. CO_2 fixation was quantified (28), and the values obtained were divided by the absorptance of the plants and normalized to a peak value of 1.0 at 660 nm. Light intensity was limiting for all the photosynthetic activity spectra reactions.

synthesis and degradation of 32-kDa protein are also promoted by irradiation at 300 nm. At this UV_B wavelength, total photosynthetic activity is quite low, while absorptance (1 − transmittance) by the plant is quite high (Fig. 1*B*). Thus, turnover of 32-kDa protein is promoted over an unusually wide spectral range in both the presence and the absence of photosynthetic activity. Such a variety of conditions leading to a single photoresponse might indicate the involvement of more than one photosensitizer.

Spectral Response for 32-kDa Protein Degradation. Degradation of 32-kDa protein was quantified at 14 wavelengths from 254 nm to 731 nm (Fig. 2). The results are expressed as the rate of 32-kDa protein degradation per equal (limiting) photon flux of incident radiation. In far red and visible light, the shape of the degradation-rate spectrum was coincident with the absorptance spectrum (cf. Fig. 1*B*) of the bulk photosynthetic pigments (mainly chlorophyll and carotenoids). A quantum-yield response spectrum (the rate of degradation divided by relative absorptance) should produce a horizontal line wherever absorbance by the bulk photosynthetic pigment leads to degradation. Fig. 2 (*Inset*) shows that such a flat response was obtained for all investigated wavelengths from 447 nm to 731 nm. This suggests that chlorophylls and the other photosynthetic pigments are the photosensitizers for degradation in these spectral regions. However, the quantum yield of the rate of degradation in the UV_C, UV_B, UV_A, and violet regions (254–429 nm) does not follow the flat response, implying that another absorbing species acts as the photosensitizer for 32-kDa protein degradation at these lower wavelengths.

Separate Photosensitizers Mediate Degradation of 32-kDa Protein in the Visible and UV Regions. The supposition that the photosensitizers for degradation of 32-kDa protein are different in the visible and UV regions of the spectrum is supported by the effect seen on the rate of degradation by mixing UV_B radiation and visible light. In an experiment in which 1 μmol·m^{-2}·s^{-1} of 313-nm radiation was given simultaneously with 5 μmol·m^{-2}·s^{-1} of visible light, a degradation rate of 0.16 (±0.01) hr^{-1} was obtained. This is greater than the sum of the rates for these fluences given separately: <0.035 hr^{-1} at 1 μmol·m^{-2}·s^{-1} of 313-nm radiation and 0.075 (±0.005) hr^{-1} at 5 μmol·m^{-2}·s^{-1} of visible light. Thus, these results indicate a synergistic effect. Such a synergism is evidence of more than one photosensitizer contributing to a single process (29, 30).

Additional support for the involvement of more than one photosensitizer comes from fluence response curves for the rate of degradation of 32-kDa protein under UV and visible radiation (Fig. 3). Degradation of 32-kDa protein approaches a higher saturation rate under UV radiation than in visible light. Such a difference in saturation kinetics is likewise consistent with the proposal that there are multiple photosensitizers (cf. ref. 31).

Tentative Identification of the Photosensitizers for Degradation of 32-kDa Protein. Chlorophyll is a major absorbing pigment in green plants in the UV as well as the visible region. This is due to its abundance in photosynthetic tissue and its relatively high extinction coefficient even in the UV_C and UV_B spectral regions [$\varepsilon_{250-300\ nm}$ = 20–25 mmol^{-1}·cm^{-1} (32)]. If chlorophyll is the photosensitizer for 32-kDa protein degradation in the 400- to 700-nm region, while a minor absorbing species (i.e., a molecule that is present in low abundance and/or one that absorbs weakly relative to chlorophyll) is the UV photosensitizer, then lowering the amount of chlorophyll in the plant tissue might sensitize degradation in the UV region, but it should have little effect on the rate of degradation in visible light. Plants given intermittent light/dark cycles of 2 min of visible light and 2 hr of darkness have <10% of antennae chlorophyll. Yet, when placed in continuous light they are capable of photosynthesis (33) and rapid turnover of 32-kDa protein (34). The 32-kDa protein degradation-rate spectrum for these intermittent light-grown plants is shown in Fig. 4 for 11 wavelengths from 254 nm to 660 nm at equal incident quanta (6 μmol·m^{-2}·s^{-1}). The comparable rates for plants grown in continuous light are indicated as well. Strikingly, enhancement is greatest at wavelengths < 447 nm, as shown by the degradation rate difference spectrum (Fig. 4 *Inset*). Thus, removal of bulk chlorophyll apparently unmasks a photosensitizer in the UV region.

The identity of a UV photosensitizer for degradation of 32-kDa protein is suggested by the shape of the degradation-

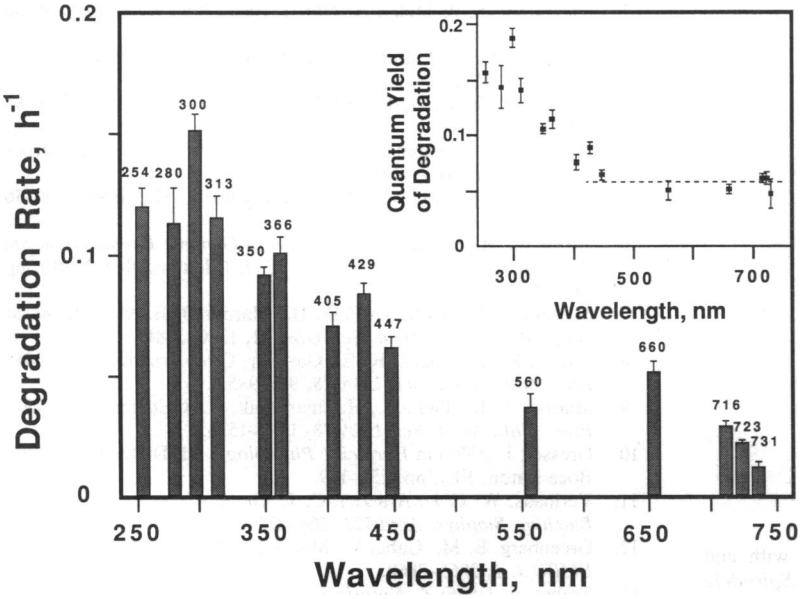

FIG. 2. *In vivo* action spectrum for degradation of 32-kDa protein. *Spirodela* plants were radiolabeled and chased as described for degradation of 32-kDa protein in the legend to Fig. 1. Membrane protein samples were fractionated by SDS/PAGE (26) and fluorograms were quantified by densitometry (12). The data were normalized by dividing the 32-kDa protein peak height by the light-harvesting chlorophyll *a*/*b* binding protein (LHCP) peak height. LHCP is a stable protein under our experimental conditions (cf. Fig. 1*A* and refs. 6 and 15). The rate of degradation of 32-kDa protein for equal quanta of incident radiation (6 μmol·m^{-2}·s^{-1}) is given for 14 different wavelengths. At all wavelengths tested, the kinetics of degradation of 32-kDa protein followed an exponential decay curve (data not shown). Therefore, the half-life, plotted as $1/t_{1/2}$ was used to measure the degradation rate. Error bars are SEM (n = 6–35). (*Inset*) The quantum yield of the degradation rate, given as the ratio of the degradation response spectrum to the relative absorptance spectrum (cf. Fig. 1*B*), is shown.

rate spectra from 254 nm to 429 nm for both continuous and intermittent light-grown plants (Fig. 4). The patterns are similar to the published absorbance spectra for plastoquinone in its various forms (19, 35). During photosynthetic electron transport, the exchangeable plastoquinone attached to 32-kDa protein (36) goes through three redox states: quinone, semiquinone anion radical, and quinol (17, 18). As shown in Fig. 4 (*Inset*), the absorbance spectrum for plastosemiquinone anion radical (19) resembles the 32-kDa protein degradation-rate difference spectrum. The spectra for other ligands associated with 32-kDa protein [e.g., chlorophyll *a* (32) and pheophytin (37)] do not show any such resemblance in the UV region.

The action spectrum for degradation of 32-kDa protein reflects the absorbance spectra of all species contributing to the various steps of the process. While our data are consistent with plastoquinone being a major contributor in the UV region, it is important to note that other UV-absorbing species, particularly amino acid radicals (38), may also be involved.

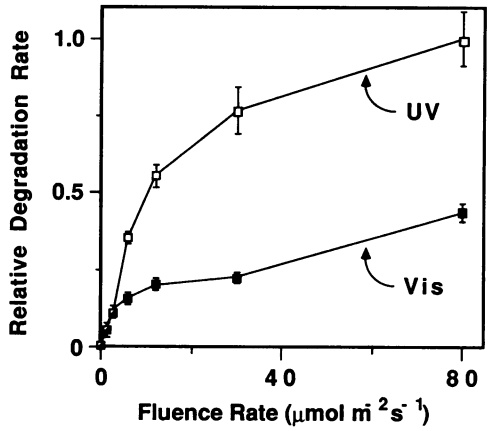

FIG. 3. Dependence of degradation of 32-kDa protein on fluence rate. The rate of degradation of 32-kDa protein was determined as a function of fluence rate in the UV and visible regions. Pulse labeling, chase conditions, and quantification of data were as described for degradation of 32-kDa protein in the legends to Figs. 1 and 2. Visible (Vis) light was generated from cool-white fluorescent bulbs. UV radiation was generated with a 250-W Hg lamp fitted with a UG11 filter (Schott), resulting in the isolation of the 313-nm, 334-nm, and 366-nm emission bands (80 μmol·m^{-2}·s^{-1} was the highest fluence rate obtained with this configuration). Rates are given in relative units with the value at 80 μmol·m^{-2}·s^{-1} UV radiation ($1/t_{1/2}$ = 0.52 hr^{-1}) being set equal to 1. Error bars represent SEM (n = 6–9).

Contributions by such species could help explain local mismatches between the 32-kDa protein degradation-rate spectrum and the plastosemiquinone anion radical absorbance [particularly from 300 nm to 320 nm where, for example, tyrosine radical absorbs (39)]. It is also likely that oxygen, with which Q_B can react (40, 41), plays a part in the cleavage of the

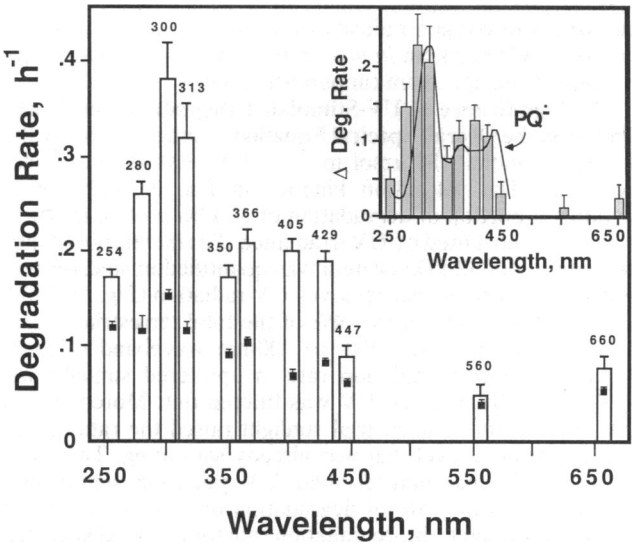

FIG. 4. Enhanced degradation of 32-kDa protein in the UV region in antennae chlorophyll-deficient plants. Degradation of 32-kDa protein was assayed in *Spirodela* plants cultured under intermittent light/dark cycles of 2 min of 50 μmol·m^{-2}·s^{-1} visible light followed by 2 hr of darkness. The chlorophyll content of these plants was 0.09 mg per mg of fresh weight versus 1.40 mg per mg of fresh weight for plants cultured in continuous light. The intermittent light-grown plants were transferred to continuous light and immediately radiolabeled with [^{35}S]methionine for 2 hr in 25 μmol·m^{-2}·s^{-1} visible light. The radioactivity was chased and the rate of 32-kDa protein degradation was determined as described in the legends to Figs. 1 and 2. Radiation conditions for the chase experiments were as described in the legend to Fig. 2. The rates of 32-kDa protein degradation in intermittent light-grown plants are represented by histograms: solid squares represent the rate in continuously illuminated plants. Error bars are SEM (n = 6–20). (*Inset*) The difference spectrum for degradation of 32-kDa protein (Δ degradation rate), shown as histograms, was obtained by subtracting the 32-kDa protein degradation rates of the continuously illuminated plants from those of the intermittent light-grown plants. The absorbance spectrum of the plastosemiquinone anion radical ($PQ^{\cdot-}$) (——) is taken from Amesz (19).

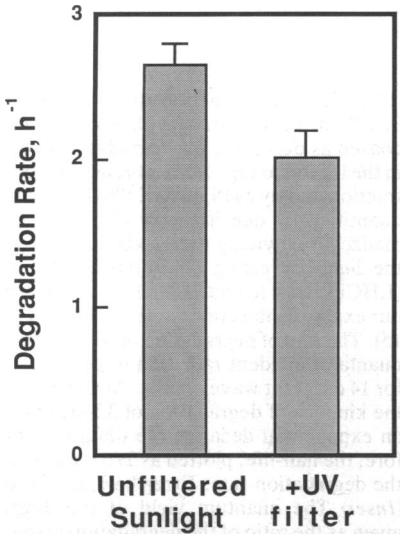

FIG. 5. Degradation of 32-kDa protein in sunlight with and without a UV filter. Radiolabeling and chase conditions for *Spirodela* were as described for degradation of 32-kDa protein in the legend to Fig. 1. Radiation conditions for the chase experiments were sunlight at noon either unfiltered (1400–1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$) or filtered to remove all detectable UV light (GG400 filter, Schott; 1320–1420 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Samples were removed at 0, 15, and 30 min of chase. Membrane proteins were analyzed and quantified as described in the legend to Fig. 2. Error bars are SEM ($n = 9$).

32-kDa protein peptide chain. In a complementary study, quenchers of oxygen radicals were found to inhibit degradation of 32-kDa protein *in vivo* in the UV, visible, and far red regions of the spectrum (unpublished data).

The Significance of UV-Stimulated Degradation of 32-kDa Protein in the Overall Spectral Equation. Sunlight at the earth's surface contains ≈ 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ UV (300–400 nm) radiation (42). The saturation kinetics in Fig. 3 imply that a significant portion of degradation of 32-kDa protein in nature might be contributed by UV irradiance. To test this possibility, degradation of 32-kDa protein was quantified in sunlight with and without a filter that removes UV radiation (Fig. 5). While UV radiation contributes $\approx 4\%$ of the total terrestrial photon flux of sunlight in the 300- to 700-nm waveband (42), the 32-kDa protein degradation rate in unfiltered sunlight was $\approx 30\%$ faster than when UV was filtered out. Moreover, the UV component of terrestrial sunlight raised the rate of degradation from a level that was already saturating. Therefore, we conclude that natural solar UV radiation significantly contributes to the rate of degradation of 32-kDa protein. In unfiltered sunlight, the degradation rate for the 32-kDa protein ($1/t_{1/2}$) is $>2.6\ hr^{-1}$ (Fig. 5). This translates to a half-life of <25 min, which places 32-kDa protein among the most rapidly turning over proteins (cf. ref. 14).

The rate of degradation of 32-kDa protein peaks in the UV_B region (280–320 nm) of the spectrum. This region is effectively screened by the ozone layer of the stratosphere (43, 44). Thus, UV-activated turnover of 32-kDa protein may have ecological implications, with possible deleterious effects if depletion of atmospheric ozone should occur.

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